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ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation

(protein/breakdown/energy requirement/covalent linkage of polypeptides)

AARON CIECHANOVER*, HANNAH HELLER*, SARAH ELIAS*, ARTHUR L. HAAS†, AND AVRAM HERSHKO*§

*Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel; and †The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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ABSTRACT **The heat-stable polypeptide (APF-1) required for ATP-dependent proteolysis in reticulocytes enters into high molecular weight conjugates upon incubation with the fraction of reticulocytes that is retained by DEAE-cellulose. Conjugate formation requires ATP and Mg^{2+} and is inhibited by *N*-ethylmaleimide. UTP and GTP are inactive. These properties are identical to those of ATP-dependent protein breakdown in the same system, suggesting that the conjugates are intermediates in this process. The APF-1 conjugates are stable in sodium dodecyl sulfate/polyacrylamide gel electrophoresis and Sephadex G-75 isolation and are resistant to mild acid, alkali, heat denaturation, and reduction; the conjugates are therefore covalent.**

Although most cellular proteins turn over rapidly, the enzymic reactions of protein degradation have not yet been identified. A basic feature of intracellular protein breakdown is its absolute requirement for cellular energy. Inhibitors of ATP production inhibit the degradation of liver proteins (1, 2), of tyrosine aminotransferase in hepatoma cells (3), of normal proteins in bacteria (4, 5) and cultured cells (6, 7), and of abnormal proteins in *Escherichia coli* (8, 9) and reticulocytes (10, 11). Recently several reports have shown an ATP requirement for protein breakdown in cell-free systems. ATP stimulates the degradation of abnormal proteins in crude soluble extracts of reticulocytes (10, 11) and *E. coli* (12), and a requirement for ATP has been found in the cleavage of bacteriophage λ repressor *in vitro* (13, 14).

We have shown that the ATP-dependent proteolytic system from rabbit reticulocytes is composed of several required components. A heat-stable polypeptide of a relatively small size, ($M_r \approx 8000$) designated ATP-dependent proteolysis factor 1 (APF-1), has been resolved (15). APF-1 has no proteolytic activity itself but stimulates ATP-dependent protein breakdown by a crude protein fraction eluted from DEAE-cellulose, fraction II (15). Fraction II has been resolved into two subfractions, both required for protein breakdown by the ATP–APF-1 system (16).

To gain a better insight into the roles of the different factors and of ATP in protein breakdown, we have now purified APF-1, radiolabeled it, and observed its association with other reticulocyte components. ATP is required for binding of APF-1 to reticulocyte proteins and the binding seems to be covalent in nature.

METHODS

Preparation of Enzyme Fractions and Purification of APF-1. Lysates from ATP-depleted rabbit reticulocytes were prepared and separated on DEAE-cellulose into fraction I (unadsorbed material) and fraction II (0.5 M KCl eluate), as described earlier (15, 16). Fraction I was subjected to heat treatment (90°C, 20 min) and gel filtration on a column (1.5 × 90 cm) of Sephadex G-75, as described (15). Then 10 mg of this material was adsorbed onto a column (1.5 × 30 cm) of CM-Sephadex equilibrated with 10 mM potassium phosphate and was eluted with a 0–150 mM linear gradient of KCl. The active peak, designated APF-1, eluted at about 65 mM KCl. By this three-step procedure, an apparently homogeneous preparation of the heat-stable polypeptide was obtained as indicated by the following criteria: (*i*) the specific activity of APF-1 remained constant throughout the CM-Sephadex peak; (*ii*) only the single band of APF-1 could be detected when increasing amounts of the purified polypeptide were analyzed by Na-DodSO$_4$/polyacrylamide gel electrophoresis in a 15–30% acrylamide gradient (Fig. 1). Further details on the purification and characterization of APF-1 will be described in a subsequent report.

Iodination of Purified APF-1. Pure APF-1 (750 μg) was dissolved in water and added to 1 mCi (3.7×10^7 becquerels) of carrier-free Na^{125}I and 5 μmol of potassium phosphate (pH 7.5) in a total volume of 25 μl; 5 μl of chloramine-T (2 mg/ml) was added and the sample was stirred vigorously at room temperature for 40 sec. The reaction was terminated by the addition of 5 μl of sodium metabisulfite (2 mg/ml), and the preparation was passed through a column of Sephadex G-25 (0.9 × 60 cm). The initial specific radioactivity of different preparations of ^{125}I-APF-1 was $2.7–7.7 \times 10^5$ cpm/μg, and the content of unreacted iodide varied between 10 and 30% of the total radioactivity.

Assay of Binding of ^{125}I-APF-1 to Macromolecular Components. Unless otherwise stated, the reaction mixture contained in a final volume of 50 μl: 100 mM Tris·HCl (pH 7.6), 2 mM dithiothreitol, 5 mM $MgCl_2$, 130 μg of protein of fraction II, 0.8 μg of ^{125}I-APF-1, and added nucleotides as specified. After incubation at 37°C for 30 min, the samples were separated on columns of Sephadex G-75 (0.8 × 30 cm). Unless otherwise specified, the columns were equilibrated and eluted with buffer A (20 mM Tris·HCl, pH 7.6/1 mM dithiothreitol/5 mM $MgCl_2$/1 mg of bovine serum albumin per ml) that also contained the nucleotide tested at a concentration similar to that used for incubation. Bovine serum albumin was included in the above elution buffer in order to prevent the nonspecific adsorption of the labeled polypeptide. Columns were developed at room temperature or at 4°C, as indicated in the figures. Approximately 0.6-ml fractions were collected and radioactivity was estimated in 0.2-ml samples. Under these conditions, three peaks of radioactive material were separated: Bound ^{125}I-APF-1 was eluted at the void volume; this was followed by free ^{125}I-

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Abbreviations: APF-1, APF-2, ATP-dependent proteolysis factors 1 and 2.

§ To whom correspondence should be addressed.

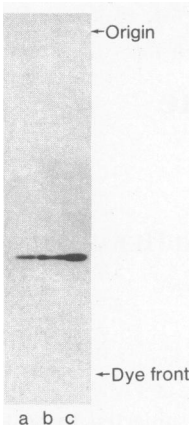

FIG. 1. Polyacrylamide gel electrophoresis of APF-1 in $NaDodSO_4$ in a gradient of 15–30% acrylamide. Purified APF-1 (after CM-Sephadex chromatography) was analyzed in the following amounts: lane a, 1.5 μg; lane b, 3 μg; lane c, 7 μg.

APF-1; the last peak was iodide ion present in the original preparation of labeled peptide. The percentage of ^{125}I-APF-1 in complex was estimated by the measurement of the areas under the peaks, and was expressed as the fraction of total ^{125}I-APF-1 radioactivity (excluding the radioactivity of free iodide).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis was performed on slab gels (13 × 13 × 0.12 cm) with the system of Laemmli (17). The concentrations of acrylamide used are noted in the legends. The gels were stained with Coomassie blue, destained with methanol/acetic acid/water (5:1:16, vol/vol), dried, and autoradiographed with Kodak X-Omat R film.

RESULTS

ATP-Dependent Binding of ^{125}I-APF-1 to Components of Fraction II. To examine the possible association of the heat-stable polypeptide with other cellular proteins and the role of ATP in this process, ^{125}I-APF-1 was incubated with fraction II in the presence or absence of ATP, and was analyzed on columns of Sephadex G-75. On such columns the low molecular weight polypeptide is retained, while most of the proteins of fraction II are excluded and eluted at the void volume (V_0). As shown in Fig. 2, subsequent to incubation of ^{125}I-APF-1 with fraction II and ATP, most of the free polypeptide disappeared, with a corresponding increase of labeled material eluted at the void volume. Without ATP, only a small part of labeled APF-1 was found in the void volume, and most of it remained in the position of free polypeptide. The third peak of low molecular weight material did not increase appreciably under these incubation conditions, which shows that ^{125}I-APF-1 itself is not hydrolyzed appreciably by the ATP-dependent system under these conditions. Control experiments showed that the presence of fraction II was absolutely necessary for the shift of ^{125}I-APF-1 to high molecular weight material, and thus the phenomenon was not due to the aggregation of the polypeptide caused directly by ATP. It seemed that the new labeled species resulted from ATP-dependent association of APF-1 with macromolecular components in fraction II that were stable in the analysis.

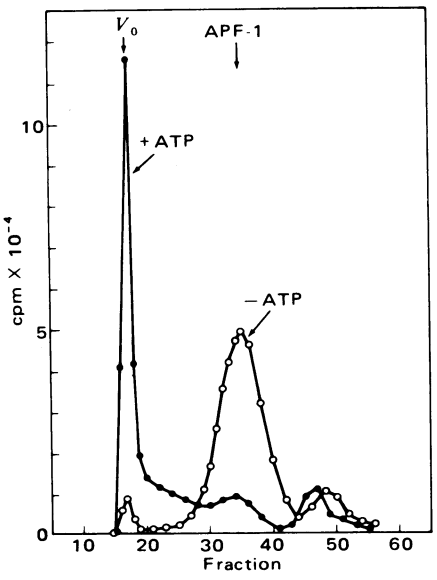

FIG. 2. ATP-dependent binding of APF-1 to reticulocyte components. Binding was assayed as described in *Methods* except that the reaction mixture contained 650 μg of fraction II and 3.2 μg of ^{125}I-APF-1 (3.8×10^5 cpm/μg). Where indicated, 2 mM ATP was present. The dimensions of the Sephadex G-75 columns were 0.9 × 55 cm, and 0.8-ml fractions were collected.

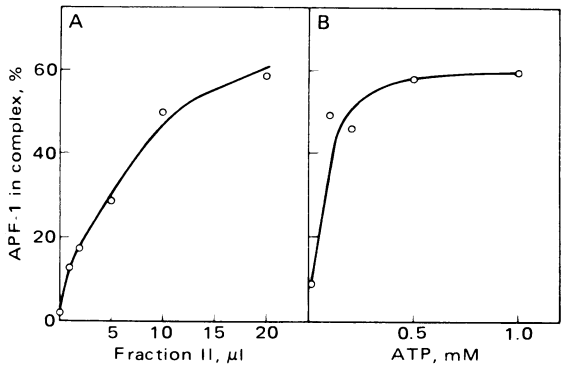

FIG. 3. Influence of the concentrations of ATP and fraction II on the binding of APF-1. (*A*) Increasing amounts of fraction II (13 mg of protein per ml) were incubated in the presence of ^{125}I-APF-1 (1.6 μg) and ATP (2 mM). (*B*) ATP at increasing concentrations was incubated with fraction II (260 μg) and ^{125}I-APF-1 (1.6 μg).

Requirements for Conjugate Formation. Fig. 3 shows the influence of increasing concentrations of fraction II and ATP on the formation of high molecular weight forms of APF-1. Conjugate formation increased with the amount of fraction II until a maximal value was attained, which varied in different experiments from 60 to 80% of total APF-1 radioactivity (as compared to a variation of 5–15% APF-1 bound in the absence of ATP). The amounts of fraction II required were similar to the amount required for ATP-dependent protein degradation

Table 1. Effects of nucleotides and inhibitors on the binding of APF-1

Addition	APF-1 bound, %
Experiment 1	
ATP	74.7
GTP	17.8
UTP	17.4
Experiment 2	
ATP	78.6
ATP plus hexokinase and glucose	14.1
ATP minus Mg^{2+}	14.0
ATP plus EDTA (5 mM)	6.6
ATP plus *N*-ethylmaleimide (5 mM)	18.3

In experiment 1, all nucleotides were tested at 0.3 mM. In experiment 2, the complete mixture contained 2 mM ATP and 5 mM $MgCl_2$. Where indicated, yeast hexokinase (Boehringer, 0.2 unit) and glucose (10 mM), or the inhibitors were added.

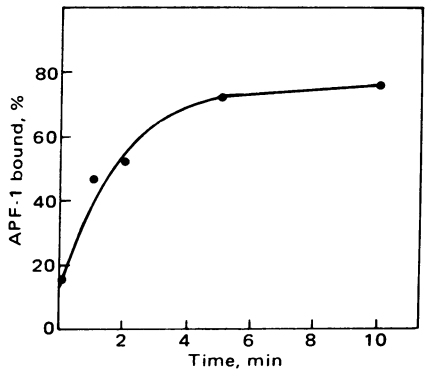

FIG. 4. Time course of association. ATP (2 mM) was present during assay and in the buffer used for column elution. At various times, the binding reaction was stopped by chilling and the sample was separated immediately on a Sephadex G-75 column at 4°C.

(15, 16). Conjugate formation was stimulated by relatively low concentrations of ATP: half-maximum complex formation was attained with less than 0.1 mM ATP (Fig. 3*B*).

The specificity of the nucleotide requirement for conjugate formation and the influence of inhibitors of the ATP-dependent proteolytic system were examined in the experiments shown in Table 1. The effect is largely specific for ATP, and little if any stimulation was found with GTP or UTP. This corresponds to earlier observations on protein breakdown (16). The addition of hexokinase and glucose abolished the effect of ATP, indicating a requirement for the terminal phosphate of the nucleotide. Mg^{2+} was required. The sulfhydryl blocking agent *N*-ethylmaleimide, which inhibits ATP-dependent protein breakdown (11), also inhibited ATP-stimulated conjugate formation.

Kinetics of the Binding and Dissociation of APF-1. Formation of APF-1 conjugates was strongly temperature dependent. At 4°C for 60 min, only about 15% of the polypeptide became bound. At 37°C, the binding was quite rapid: half-maximal levels of association were reached after approximately 1.5 min, and the binding was essentially complete by 5 min (Fig. 4). By contrast, the time course of the dissociation of the complex displayed a composite kinetic behavior (Fig. 5). In these experiments ^{125}I-APF-1 was first allowed to interact with fraction II components in the presence of ATP, ATP was then removed with hexokinase and glucose, and the rate of release of free APF-1 was followed by separation on G-75 columns. As shown in Fig. 5, the dissociation of the conjugates was initially

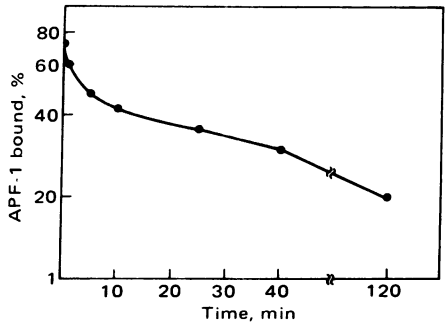

FIG. 5. Time course of dissociation of the complex. ^{125}I-APF-1 was incubated with fraction II and ATP (2 mM) for 10 min, and then 1.2 units of yeast hexokinase (Worthington) and 10 mM glucose were added and the incubation was continued at 37°C. At various times, samples were withdrawn and separated on Sephadex G-75 columns at 4°C. The zero-time sample was separated with buffer A containing 1 mM ATP, whereas the other samples were separated without ATP.

rapid ($t_{1/2} \approx 1$ min), but this was followed by a much slower phase and it required an incubation of 2 hr to obtain a nearly complete dissociation. This suggested that the APF-1 conjugates are heterogeneous.

Characterization of the Conjugation Linkage. ^{125}I-APF-1 was incubated with fraction II and ATP and was subsequently treated with various denaturing agents. As shown in Fig. 6, treatment with 0.1 M NaOH for 15 min decreased the amount of high molecular weight ^{125}I-APF-1 conjugates by about 30%. Further incubation with 0.1 M NaOH at 37°C for 60 min did not cause any further decrease in the amount of bound APF-1 (data not shown), and thus it seems that the initial decrease may be due to the disruption by alkali of a noncovalent complex that contains APF-1, while the rest is bound by a rather stable linkage. The stability to alkali also suggested that the macromolecule bound to APF-1 is protein rather than RNA. The alkali-stable residue did not dissociate even when separated on Sephadex G-75 columns equilibrated with 1 M formic acid (Fig. 6*B*) or 6 M guanidine hydrochloride (not shown).

In order to determine to which reticulocyte proteins APF-1 becomes stably bound, a reaction mixture was analyzed on $NaDodSO_4$/polyacrylamide gels (Fig. 7). ^{125}I-APF-1 radioactivity was found in many bands of the gel, indicating a wide size distribution. This distribution was unchanged if the samples were heated at 100°C for 10 min or if they were heated with extremely high concentrations of $NaDodSO_4$ (2.5%) or mercaptoethanol (5.0%); the latter result ruled out the possibility of a disulfide linkage.

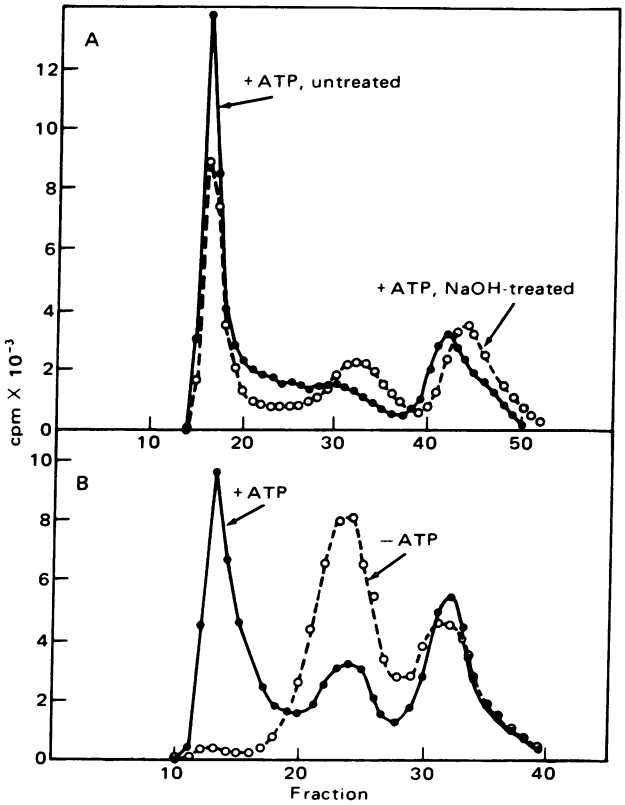

FIG. 6. Resistance of the bond to alkali and acid. (*A*) ^{125}I-APF-1 was incubated with fraction II and ATP (2 mM) for 10 min and then 0.1 M NaOH was added and the sample was incubated at 37°C for a further 15 min. Both untreated and NaOH-treated samples were separated on columns equilibrated with buffer A containing 1 mM ATP. (*B*) ^{125}I-APF-1 was incubated in the presence or absence of ATP and then treated with 1 M formic acid for 15 min. Both samples were separated on Sephadex G-75 columns equilibrated with 1 M formic acid and containing 1 mg of bovine serum albumin per ml.

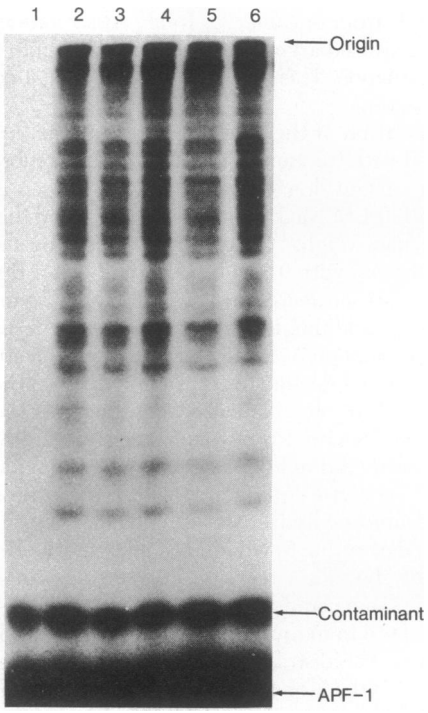

FIG. 7. Resistance of the bond to heat denaturation and reduction. ^{125}I-APF-1 (3.5 μg, 80,000 cpm) was incubated with fraction II without ATP (track 1) or in the presence of 2 mM ATP (tracks 3–6) and subsequently treated as follows: heated at 100°C with 0.5% Na-DodSO$_4$ and 0.5% 2-mercaptoethanol for 2 min (track 2), 5 min (track 3), or 10 min (track 4); heated at 100°C for 2 min with 5% (vol/vol) mercaptoethanol and 0.5% NaDodSO$_4$ (track 5) or with 2.5% Na-DodSO$_4$ and 0.5% mercaptoethanol (track 6). The samples were separated on 12.5% polyacrylamide gels and autoradiographed. Autoradiography revealed a trace contamination in ^{125}I-labeled APF-1 which was not detected by dye in the unlabeled polypeptide.

DISCUSSION

This work was initiated after several preliminary observations suggested that there is an ATP-dependent interaction between APF-1 and other components of the reticulocyte system. A difference in the requirement for APF-1 in different preparations of fraction II had been observed, which depended on how fraction II was prepared. When prepared from lysates derived from ATP-depleted reticulocytes (15, 16), as usual, ATP-dependent protein breakdown was greatly stimulated by the addition of APF-1 (15) and ATP had little, if any, effect in the absence of the heat-stable polypeptide. When fraction II was prepared from untreated reticulocytes (containing endogenous ATP), stimulation of protein breakdown was obtained by ATP in the absence of added APF-1 and the effect of added APF-1 was much less (unpublished results). One possible interpretation of this observation was that, in the normal cell, APF-1 is present in conjugates that remain intact through the purification on DEAE-cellulose. The bound form of APF-1 could then replace the requirement for free APF-1 if this was generated by breakdown of the complexes (Fig. 5). Another observation was that the content of free APF-1 in extracts of tissues such as the liver was markedly increased after the depletion of ATP (unpublished results). The finding of stable conjugates seems to provide an explanation for the above observations.

It seems reasonable to assume that the observed association is relevant to the roles of APF-1 and ATP in protein breakdown. The amounts of ATP, APF-1, and fraction II required for complex formation and for protein breakdown are comparable. Furthermore, both processes show essentially the same nucleotide specificity, and both are inhibited by EDTA and *N*-ethylmaleimide (Table 1), which also inhibit protein breakdown (11).

The possibility that an enzymatic reaction is involved in the ATP-dependent reaction of APF-1 with reticulocyte proteins was suggested by the findings that conjugate formation is time and temperature dependent (Fig. 4) and that it is blocked by *N*-ethylmaleimide (Table 1). That a covalent bond might be formed was also strongly suggested by the slowness of the dissociation of the conjugates (Fig. 5). It was surprising to find, however, that a stable bond links the heat-stable polypeptide directly to reticulocyte proteins (Figs. 6 and 7). It is possible that besides the covalently bound polypeptide, another smaller part is associated in a noncovalent complex (Fig. 6). It appears reasonable to assume that the noncovalent complex represents the initial interaction between APF-1, ATP, and some enzyme(s), and that the covalently bound compounds are products of ensuing reactions.

The finding that numerous reticulocyte proteins are bound covalently to APF-1 (Fig. 7) appears to be incompatible with the notion that the linkage is to specific proteins. It seems rather that various endogenous (possibly denatured) reticulocyte proteins, which are substrates for the ATP-dependent reticulocyte system, are the targets of the covalent binding. In that case, it will have to be concluded that the ATP-requiring covalent linkage of the heat-stable polypeptide to the substrates is part of the mechanism by which APF-1 participates in protein breakdown. In the subsequent article, it will be shown that the heat-stable polypeptide is in fact linked to proteins that are good substrates for ATP-dependent proteolysis reaction (18).

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